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The Most Prevalent Hepatitis C Virus Genotypes in England and Wales Are 3a and 1a

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Hepatitis C virus (HCV) genotypes were assigned to 567 individuals by restriction fragment length polymorphism analysis of the 5′ noncoding region of the HCV genome following reverse transcription–polymerase chain reaction. The groups of individuals in this study included hemophilia patients, injecting drug users (IDUs), blood donors, antenatal patients, those attending genitourinary medicine (GUM) clinics, and patients with chronic liver disease, all from England and Wales. The majority of HCV infections were types 1a (32%), 1b (15%), or 3a (37%). The genotype distribution in individual groups was similar to the overall genotype distribution except for hemophilia patients, in whom the frequencies were 1a (39%), 1b (23%), and 3a (21%). With the exception of hemophilia patients, subpopulations in England and Wales appear to share common modes of HCV transmission. There is a need for continued surveillance to monitor the spread of possibly more virulent or drug-resistant HCV genotypes. ***J. Med. Virol. 58:127–131, 1999.*** © 1999 Wiley-Liss, Inc.

KEY WORDS: polymerase chain reaction; restriction fragment length polymorphism; injecting drug use; hemophilia; interferon

INTRODUCTION

The hepatitis C virus (HCV) genome displays sequence diversity. Variants can be classified into six major genotypes, each of which contains a number of closely related subtypes [Simmonds et al., 1993]. Different genotypes may carry different clinical significance [Dusheiko and Simmonds, 1994; Bruno et al., 1997; Kiyosawa, 1997; Mihm et al., 1997]. They may also influence response to interferon-alpha, currently the only therapeutic drug licensed for the treatment of HCV infection [Dusheiko and Simmonds, 1994; Dusheiko et al., 1996; Isaacson et al., 1997; Kiyosawa, 1997].

The predominant genotypes in Europe are types 1, 2, and 3. In northwestern Europe, types 1a, 1b, and 3a occur most frequently [Pohjanpelto et al., 1996; Berg et al., 1997]. In southern European countries, genotype 1 is also prevalent, as is genotype 2 [Guadagnino et al., 1997; Lopez-Labrador et al., 1997]. In the Far East, the most common genotypes are 1b and 2 [Wu et al., 1997], while in North America, genotypes 1a and 1b prevail [Zein et al., 1996]. Of the other genotypes, type 3b is commonly found in the Indian subcontinent, type 4 in North Africa and the Middle East, and type 5 in South Africa. Type 6 is predominant in the Far East, particularly Hong Kong, Macau, and Vietnam [Dusheiko et al., 1994; Davidson et al., 1995; McOmish et al., 1994; Mellor et al., 1995]. Some researchers have reported the existence of genotypes 7, 8, and 9 in Vietnam [Tokita et al., 1994, 1995]. Furthermore, in a given geographical region different genotypes may predominate in different subpopulations [Pawlotsky et al., 1995; Basaras et al., 1997]. Such subpopulations would include groups of people at high risk for acquiring HCV infection, e.g., hemophilia patients or injecting drug users (IDUs). Immigrants can also bring with them HCV genotypes peculiar to their country of origin.

In England and Wales, the extent to which HCV genotypes differ in at-risk groups has not been investigated until now. Prospective knowledge of this information is required to determine what the current range of genotypes is. This will allow more informed management of infection [Dusheiko et al., 1996] and facilitate earlier recognition of outbreaks caused by new or unusual genotypes. A systematic study of prevailing genotypes in England and Wales was carried out and the results were compared with international data.

MATERIALS AND METHODS

Patients

Between August 1996 and March 1998, samples from 567 HCV RNA-positive individuals, referred from 46

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centers, were genotyped. Samples were from 77 hemophilia patients, 68 screened blood donors, and 78 IDUs. Other samples were from 62 antenatal clinic (ANC) patients and 86 genitourinary medicine (GUM) clinic patients identified in the Unlinked Anonymous Seroprevalence Monitoring Programme [Unlinked Anonymous HIV Surveys Steering Group, 1996]. The remaining samples were from 194 individuals investigated for liver disease. The six patient groups were analyzed by sex and age, <40 years and ≥40 years, with the exception of the GUM group, where a cutoff of 35 years was used.

Amplification of HCV 5′ Noncoding Region

RNA was extracted from 100 μl of sera or plasma using the Amplicor HCV Specimen Preparation Kit (Roche Diagnostic Systems, Welwyn Garden City, Herts, UK). The final pellet was resuspended in 50 μl of nuclease-free water. RNA was reverse-transcribed using Murine Maloney Leukemia Virus reverse transcriptase (Life Technologies, Paisley, UK) and random hexamers. The HCV 5′ noncoding region (5′-NCR) was amplified by nested PCR. Primary amplification was carried out using 2-mM $MgCl_2$, 1 mM each of the four dNTPs, 5 pmoles of sense primer 57 and of antisense primer 321, and 0.625 units of Taq polymerase (Life Technologies, Paisley). Reactions were heated to 94°C for 30 sec followed by 35 cycles of 94°C for 30 sec, 62°C for 40 sec, and 72°C for 50 sec.

Secondary amplification was from 2 μl of primary PCR product under identical conditions, except that 25 pmoles of sense primer 126 and 25 pmoles of antisense primer 299 were used and cycling conditions were as follows: 94°C for 30 sec, 68°C for 40 sec, and 72°C for 30 sec. Primer sequences were as described by Lin et al. [1992]: 57 (sense), 5'-AGCGTCTAGCCATGGCGT; 321 (antisense), 5'-GCACGGTCTACGAGACCT; 126 (sense), 5'-GTGGTCTGCGGAACCGG; 299 (antisense), 5'-GGGCACTCGCAAGCACCC.

Restriction Digests of Second-Round PCR Product

The 174 base pair second-round PCR product was digested with *ScrfI, MvaI, HinfI,* and *BstUI* [Pohjanpelto et al., 1996] in four individual 10-μl reactions using 4.5 μl of PCR product. Following incubation at 37°C for 1.5 hr, 60°C for *BstUI,* digested samples were heated to 80°C for 15 min to denature the enzymes. *ScrfI, HinfI,* and *BstUI* were supplied by New England Biolabs (Hitchin, Herts, UK) and *MvaI* by Boehringer Mannheim–Hoffman La Roche (Welwyn Garden City, Herts, UK). Digests were electrophoresed through an MDE gel (Flowgen, Lichfield, Staffordshire, UK). *ScrfI* and *MvaI* digests were loaded into one well and *HinfI* and *BstUI* digests into the contiguous well. A 48-well shark tooth comb was used for loading. The gels were run at 170 V for 3 hr, then stained with SYBR-GREEN I (Flowgen) and visualized by ultraviolet transillumination.

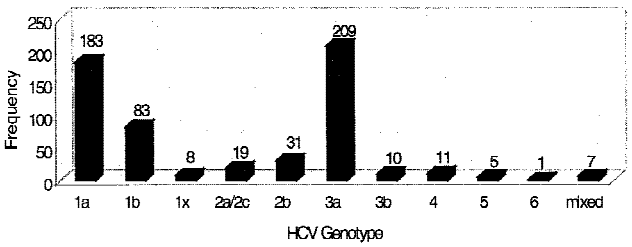

Fig. 1. HCV genotype distribution for 567 individuals in England and Wales determined by PCR RFLP between August 1996 and March 1998.

Sequencing

Samples whose genotypes could not be determined by PCR and restriction fragment length polymorphism (RFLP) analysis were sequenced with primers 126 and 299. Second-round PCR products were purified using the Igenie DNA extraction kit (Immunogen, Sunderland, Tyne and Wear, UK). Sequencing reaction were performed using the ABI Prism DNA sequencing kit (PE Applied Biosciences, Warrington, UK) and reactions were run on the ABI 373 automated sequencer. Sequences were aligned and phylogenetically analyzed with other HCV 5'NCR sequences by the CLUSTAL algorithm in the MEGALIGN program of the LASERGENE system (DNASTAR, Madison, WI).

Cloning

Second-round PCR products of some samples were cloned using the Ingenius LigATor AT-cloning kit (R&D systems, Abingdon, Oxfordshire, UK). Colonies with inserts were picked and amplified directly using PCR conditions described earlier for second-round PCR. The products then underwent RFLP analysis as described previously.

Statistical Analysis

Single variable analyses [Everitt, 1997] were performed to investigate whether the genotype distributions differed for each patient group by age or sex. The genotypes were grouped into 1a, 1b, 2, 3a, 3b, 4, 5, and 6. The type 1x group and those with mixed genotypes were excluded from this analysis. Log-linear regression analysis was performed in order to investigate any associations between genotype distribution and age, sex, or patient group.

RESULTS

The distribution of HCV genotypes infecting 567 individuals in this study is shown in Figure 1. Genotype distributions for hemophilia patients, blood donors, IDUs, ANC attenders, GUM clinic attenders, and individuals with liver disease are displayed in Figure 2. Among hemophilia patients, the most common genotypes were 1a (39%), 1b (23%), and 3a (20%). This distribution differed from the other five groups, all of which were similar to the overall genotype distribution.

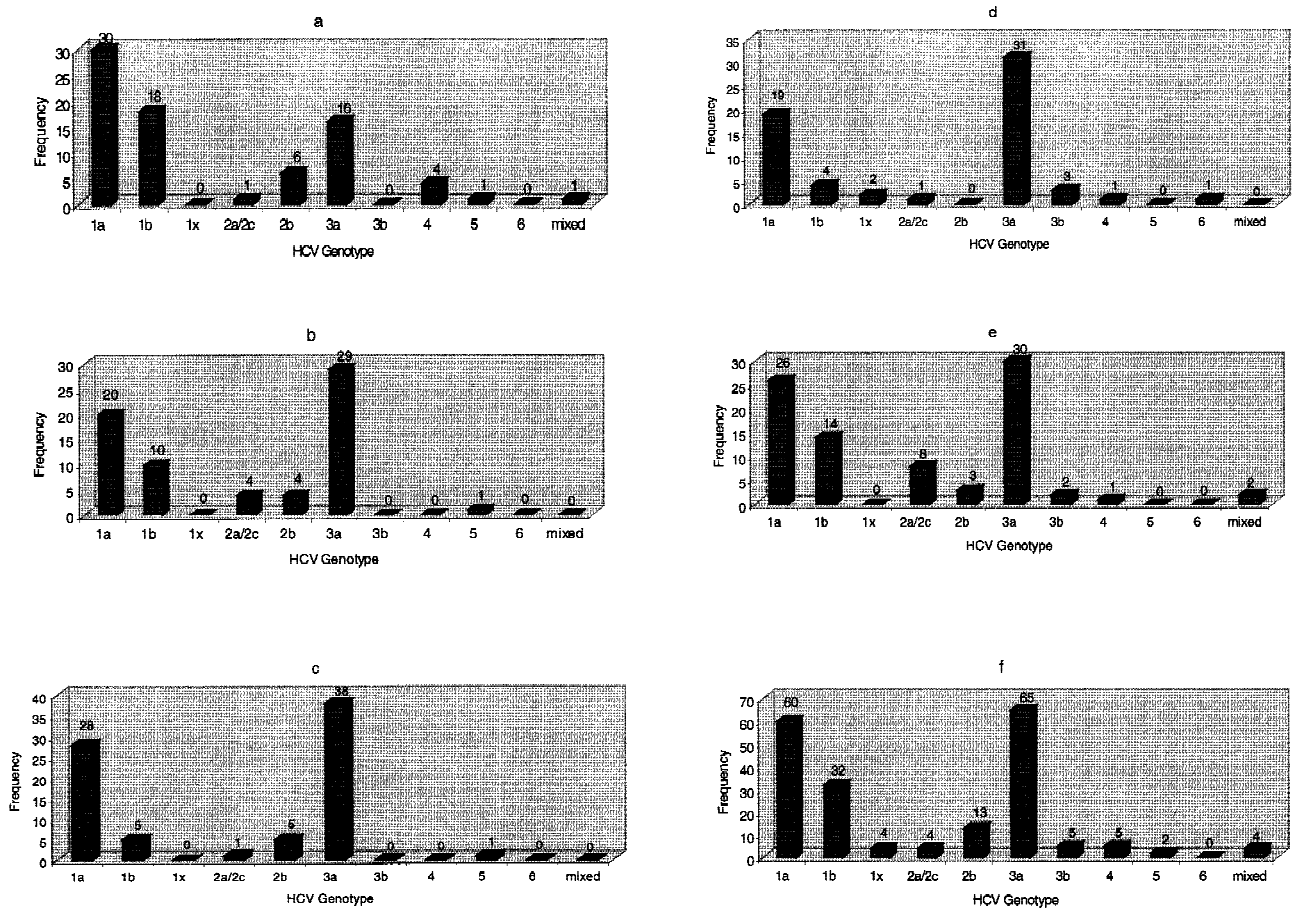

Fig. 2. HCV genotype distribution for six groups: 77 hemophiliacs **(a)**, 68 blood donors **(b)**, 78 IDUs **(c)**, 62 antenatal clinic attenders **(d)**, 86 genitourinary medicine clinic attenders **(e)**, and 194 patients with liver disease **(f)**.

Fourteen of these samples could not be assigned genotypes by RFLP analysis alone, so the second-round PCR products of these samples were subjected to direct DNA sequencing. Following sequencing, eight individuals were found to be infected with HCV genotype 1 but an existing subtype could not be assigned (represented as type 1x in Figs. 1 and 2).

Eight samples (1%) were suspected to contain a mixture of two HCV genotypes or subtypes. Cloning the second-round PCR products of these eight samples and performing RFLP analysis on the resulting colony PCR products confirmed the presence of multiple HCV genotypes or subtypes. Four samples contained a mixture of genotypes 1a and 1b, two samples contained a mixture of types 1a, 1b, and type 1x, and one sample contained a mixture of genotypes 1b and 2b. The final sample appeared to contain a mixture of genotypes 3b and 4 following RFLP analysis of clones. However, subsequent sequencing of the clones revealed that the two species present in this sample were both genotype 4. A single base change created a *BstUI* restriction site in one species giving the 3b RFLP pattern, but the overall nucleotide sequence in this region was more closely related to type 4 sequences than 3b sequences.

The hypothesis that genotype is associated with sex and age of patients was tested by Chi-square analysis [Everitt, 1997]. Very few patients were of genotype 3b–6 and they were therefore excluded from this preliminary analysis. It was not appropriate to test for an association between sex and genotype for hemophilia patients due to the small number of females in this group. Analysis at this stage was not necessary for those attending ANCs, which comprised females all under 40 years of age. Chi-square analysis shows that there is no evidence to support the hypothesis of an association between genotype distribution and either sex or age for five of the six patient groups ($P > 0.05$). Log-linear regression analysis showed a highly significant interaction between group and genotype ($P = 0.0007$), suggesting that the genotype distribution differs significantly between patient groups. The genotype distribution among hemophilia patients, when compared to the other five groups as a whole, was significantly different ($P = 0.0023$).

DISCUSSION

Several different methods have been used to genotype HCV without resorting to DNA sequencing. They include DNA amplification with genotype-specific primers [Okamoto et al., 1992; Ohno et al., 1997; Wu et

TABLE I. Comparison of HCV Genotype Distributions in Eight Countries[a]

Country	Size of study	Risk group of subjects	Genotypes, ranked in descending order of frequency (%)					
England and Wales[b]	567	Various	3a (37)	1a (32)	1b (15)	2b (5)	2a/c (3)	Other (8)
Finland[c]	264	Various	3a (41)	1b (24)	2b (20)	1a (14)	Other (1)	
Germany[d]	379	Chronic hepatitis patients	1b (56)	3a (26)	1a (14)	2 (4)		
Spain[e]	414	Chronic hepatitis patients	1b (82)	1a (8)	3a (5)	2a/c (3)	4 (1)	
Southern Italy[f]	148	Various	1b (51)	2c (45)	3a (3)	4 (1)	2b (1)	
United States[g]	179	Various	1a (58)	1b (21)	2b (13)	3a (5)	2a (2)	4 (1)
Thailand[h]	235	Blood donors	3a (39)	1b (20)	6 (18)	1a (9)	3b (4)	Other (10)
Taiwan[i]	562	Blood donors	1b (60)	2a (16)	2b (12)	3a (2)	Mixed (7)	Other (3)

[a]Only published studies with >100 subjects are included
[b]This study.
[c]Pohjanpelto et al. [1996].
[d]Berg et al. [1997].
[e]Lopez-Labrador et al. [1997].
[f]Guadagnino et al. [1997].
[g]Zein et al. [1996].
[h]Kanistan et al. [1997].
[i]Wu et al. [1997].

al., 1997], hybridization of biotinylated PCR products to membrane-bound type-specific probes (line probe assay) [Stuyver et al., 1996], and RFLP analysis of amplified DNA [Davidson et al., 1995; Pohjanpelto et al., 1996]. Our study utilized the PCR RFLP method based on Pohjanpelto et al. [1996], modified to permit a high throughput analysis of a large number of specimens.

We found the most prevalent HCV genotypes in England and Wakes to be 3a (37%), 1a (31%), and 1b (15%). These genotypes are found in other countries, but the finding of 3a as the most common subtype suggests that the overall distribution is more similar to northern European countries (Table I). There were also a number of infections by genotypes that are not common in Europe. Only seven (1%) samples contained more than one genotype. Other groups have reported a higher incidence of infection with multiple HCV genotypes [Ohno et al., 1997; Wu et al., 1997]. A prevalence of 1% does seem low when high-risk groups such as hemophiliacs and IVUs were included in the study.

The HCV genotype distribution among hemophilia patients differed significantly from the other five groups. This could be due to infection by nonindigenous strains contaminating clotting factor concentrates imported from North America, where types 1a and 1b prevail (Table I), or it may reflect circulating genotypes at a time prior to the heat treatment of blood products. Otherwise, there is a striking similarity in HCV genotype distribution between groups, suggesting a common origin for many of these infections, possibly recreational drug use in the community [Pawlotsky et al., 1995; Basaras et al., 1997; Kiyosawa, 1997; Mihm et al., 1997; Smith and Simmonds, 1997].

The finding that type 3a is as prevalent as type 1 has an important implication for the screening for markers of HCV infection in England and Wales. Current serological assays, based on HCV genotype 1, are reported to be suboptimal for detecting antibody to other genotypes [Neville et al., 1997]. This might lead to delay in diagnosis or identification of HCV infection. Screening assays used in England and Wales should be optimal for genotype 3 as well as genotype 1.

Some HCV genotypes, particularly 1b, are considered to be more virulent and more resistant to treatment by interferon-α [Dusheiko and Simmonds, 1994b; Dusheiko et al., 1996; Bruno et al., 1997; Isaacson et al., 1997; Kiyosawa, 1997; Mihm et al., 1997; Pawlotsky et al., 1998]. Continued surveillance will monitor spread of possibly more virulent and drug-resistant HCV genotypes. This will guide funding allocations for prolonged or combined antiviral therapy and liver transplantation. Plans to link laboratory reports with risk factor and clinical information [Ramsay et al., 1998] will now be extended to include surveillance of HCV genotype.

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